MOUSE CIRCADIAN PLASMA LEPTIN AND ACTIVE GHRELIN RHYTHMS

UNDER *AD LIBITUM* AND SCHEDULED FEEDING

By

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A

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Abstract

Light is the strongest timing cue for the circadian system, but non-photic cues can also entrain the master circadian clock, i.e., suprachiasmatic nuclei (SCN). In one of our mouse line (ENTR), all mice entrain to scheduled feeding, while in another (NON-ENTR) only 4 % entrain. In order to explore key physiological pathways involved in that process, I quantified the circadian rhythms of plasma leptin and active ghrelin of these two lines of mice under a 12:12 hour light-dark cycle with *ad libitum* feeding and six hours of food availability during the light period.

Plasma active ghrelin induced opposite circadian rhythms compared to leptin, which were most pronounced under scheduled feeding when leptin was highest during and right after the food availability period; active ghrelin was highest at night when food was not available. Compared to *ad libitum* feeding, the overall concentration of leptin decreased and active ghrelin concentration increased significantly under scheduled feeding.

The plasma active ghrelin circadian rhythms of ENTR mice were more robust with higher amplitude rhythms than the NON-ENTR mice under *ad libitum* feeding and scheduled feeding. I hypothesize that the high amplitude plasma active ghrelin circadian rhythm provides a signal for the ENTR mice to entrain to scheduled feeding.

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Introduction

Without external signals, mammals can organize their physiological and behavioral rhythms with a near 24-h period (Reppert and Weaver, 2001). The master circadian clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, which coordinates circadian rhythms in other regions of the brain and peripheral organs (Weaver, 1998; Reppert and Weaver, 2001). Light is the most powerful Zeitgeber, or timing cue, which can shift the clock and entrain it to the external light/dark (LD) cycle (Roenneberg *et al.*, 2003).

Recurrent nonphotic events also can entrain circadian rhythms via mechanisms different from photic cues. Several nonphotic entraining stimuli in mammals are recognized, such as the behavioral activation or arousal induced by exposing animals to novel running wheels, sleep deprivation by intermittently shaking the cage, handling, or saline injection (Bobrzynska and Mrosovsky, 1998; Antle and Mistlberger, 2000; Glass *et al.,* 2001). Presenting such stimuli to animals maintained in constant darkness produces typical non-photic-like effects, including phase shifts during the subjective day. Different stimuli induce variations in the size of the phase shift and the time of maximum sensitivity (Caldelas *et al.,* 2005).

Scheduled feeding (SF) is one of several nonphotic signals (Mrosovsky, 1995, 1996) that can entrain physiological and behavioral rhythms (Mistlberger, 1993; Marchant and Mistlberger, 1997; Holmes and Mistlberger, 2000) as well. The entrainment to timed food has been reported in many animals, such as bees, fish,

marsupials, birds, mice, hamsters, rats, rabbits, weasels, and squirrel monkeys (Mistlberger, 1994; Stephan, 2001). These animals have the ability to anticipate the scheduled food availability by increasing their locomotor activity and core body temperature before the food is presented (Mistlberger, 1994; Stephan, 2001). These marked activities in anticipation of food time are referred to as food-anticipatory activity (FAA). FAA shows fundamental properties of biological clock controlled characteristics, including limits to entrainment and persistence in the absence of the zeitgeber during several cycles of total food deprivation (Mistlberger, 1994). FAA also includes pre-meal metabolic and hormonal changes such as elevated serum corticosterone levels (Krieger, 1974). In rats, a clear correlation has been found between FAA and the metabolic changes in the liver, including an increase in circulating free fatty acids and ketone bodies, and a drop in the level of triacylglycerides and liver glycogen (Escobar *et al.*, 1998).

Although the SCN is the master circadian clock in mammals' brain, complete lesions of the SCN do not abolish or even reduce food entrained physiological and behavioral rhythms, which suggest a separate circadian system that responds to food rather than light cues (Stephan *et al.*, 1979). This circadian system has the basic properties of a circadian oscillator. It can be coupled to environmental events, such as scheduled feeding, thereby enabling animals to adaptively respond to daily fluctuations in an environment with temporally restricted food availability (Pittendrigh, 1981). Researchers have been trying to locate the food entrainable oscillator (FEO) that generates FAA, but without conclusive results (Landry *et al.*, 2006; Mieda *et al.*, 2006).

A recent study shows that orexin neuron-ablated mice have reduced FAA, which indicates that orexin could be involved in the connection between the FEO and the central nervous system (Akiyama *et al.,* 2004). Other studies reveal evidence that neither vagotomy (Comperatore and Stephan, 1990) nor capsaicin-induced deafferentation (Davidson and Stephan, 1998) abolished FAA, which suggests that hormonal mediators may play a role in the communication of food related signals to the FEO.

Leptin and ghrelin are two peptide hormones, discovered in the 1990s, that have been shown to be the key hormones related to food intake and energy balance in mammals (Zhang *et al.,* 1994; Caro *et al.,* 1996; Kojima *et al.,* 1999; Kalra and Kalra, 2003). These hormones influence body weight, food intake, and cellular metabolism via interactions with feeding regulatory centers, which are located in the arcuate nucleus (ARC) and the paraventricular nucleus (PVN) in the hypothalamus (Cone *et al.,* 2001; Konturek *et al*., 2004).

Neuropeptide Y (NPY) pathways emanating from the ARC and terminating in the PVN are the primary common pathways regulating appetite expression (Kalra and Kalra, 2003). The ARC contains two major neuronal groups: neurons expressing the orexigenic neuropeptides, NPY and Agouti related protein (AgRP) (Mercer *et al.,* 1996), and neurons expressing proopimelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART) (Cheung *et al.,* 1997). NPY and AgRP pathways project from the ARC into the PVN where they stimulate appetite by activating Y1/Y5 and Mc4 receptors, respectively (Kalra and Kalra, 2003). Two important actions of leptin have been identified that result in appetite inhibition. One is inhibiting the activity of orexigenic

NPY/AgRP neurons and reducing the expression of NPY and AgRP. Another is activating anorectic POMC/CART neurons. Ghrelin receptors are located in the hypothalamus, particularly on NPY/AgRP neurons. Ghrelin stimulates appetite by stimulating NPY and AgRP secretion. During the past decade, research has shown the reciprocal action of the afferent hormonal signals of leptin and ghrelin that regulate NPY/AgRP secretion for the management of food and energy intake (Kalra and Kalra, 2003; Chen *et al.,* 2004).

Leptin is secreted by white adipose tissue (WAT) (Masuzaki *et al.,* 1995; Tsuruo *et al.,* 1996). Circulatory leptin conveys information to the brain about the amount of energy stored in adipose tissue and thus alters the energy homeostasis. In humans and rodents, plasma leptin concentration correlates positively with body fat content (Maffei *et al.,* 1995; Saad *et al.,* 1998). Leptin shows a distinctive daily variation, with nightly peaks and daily low values both in diurnal humans (Saad *et al.,* 1998) and nocturnal rodents (Ahren, 2000), although they have opposite feeding times.

Ghrelin is an endogenous ligand for the growth hormone-secretagogue receptor recently found in rats and humans. It is a 28-amino acid peptide that originates from the mucosa of the gastrointestinal tract and especially from the X/A-like cells in the stomach (Sakata *et al.,* 2002). Ghrelin is considered to be the afferent hormonal signal regulating digestive behavior. It may be a "meal control signal" because in both humans and animals, ghrelin displays a pulsatile secretion pattern (Korhonen and Saareha, 2005). Its concentration increases before each meal and falls within 1 hour after eating (Cummings *et al.,* 2001; Sugino *et al.,* 2002), and acute ghrelin administration stimulates eating

(Asakawa *et al.*, 2001; Broglio *et al.*, 2003; Faulconbridge *et al.*, 2003; Wren *et al.*, 2001). Ghrelin may also be an "adiposity signal" because the basal ghrelin levels correlate with adiposity, and in rats and mice, long-term administration of ghrelin increases body weight (Tschop *et al.,* 2000; Wren *et al.,* 2000; Nakazato *et al.,* 2001).

Castillo *et al.* (2004) were the first to show that entrainment to scheduled feeding in constant dark is at the level of the SCN, using mouse PERIOD 2 (mPER2) protein patterns as a phase reference point. Interestingly, this study also revealed that the ability to entrain to scheduled feeding in constant dark is mouse line specific. In one line (ENTR), all mice entrained to scheduled feeding, while in another only 4% entrained (NON-ENTR). These unique and robust responses of these mouse lines gave us the opportunity to explore key physiological pathways by which the master circadian clock in the SCN entrains to SF.

Because leptin and ghrelin play important roles in feeding homeostasis, we hypothesized that these hormones provide direct or indirect signals for entrainment of the SCN to SF. Measuring the plasma concentrations of these two hormones in mice under *ad libitum* feeding and under scheduled feeding is an important first step in elucidating mechanisms of entrainment of the master circadian clock to SF in a 12:12 LD cycle. If these hormones play a role in entrainment of the SCN to scheduled feeding in constant dark, we expected a difference in the circadian rhythms of plasma leptin and ghrelin between the ENTR and NON-ENTR mice.

Materials and Methods

Animals under *ad libitum* feeding

Seventy seven male mice (*Mus musculus*) from the ENTR line and 76 male mice from the NON-ENTR line, 50–60 days of age, were randomly taken from our mouse colony at the University of Alaska Fairbanks. Each mouse was housed individually on wood shavings in standard polycarbonate cages (21×37×14 cm; Nalgene, Rochester, NY). Thirty nine ENTR mice and 38 NON-ENTR mice were kept in an animal room with 12:12 LD cycle (lights on at 6 am–6 pm), while another 38 ENTR mice and 38 NON- ENTR mice were kept in another animal room with a reversed 12:12 LD cycle (lights on at 6 pm–6 am). All the animals were maintained at room temperature (20±2 °C) with *ad libitum* food and water. Standard mouse chow (Purina Mills, Lab Mouse Diet #5015, St. Louis, MO) was used throughout the experiment. All mice were kept in the new light-dark cycle for two weeks before they were euthanized for blood collection. Two ENTR mice died during the experiment.

Animals under scheduled feeding

Thirty six male mice from the ENTR line and 36 male mice from the NON-ENTR line, 40–50 days of age, were randomly taken from our mouse colony. All mice were kept in an animal room with a 12:12 LD cycle (lights on at 6 am–6 pm). All the animals were maintained at room temperature (20±2 °C) and individually housed in polycarbonate cages (21×37×14 cm) equipped with 24.2 cm diameter running wheels

(Nalgene, Rochester, NY) on wood shavings. Mouse chow (Purina Mills, Lab Mouse Diet #5015, St. Louis, MO) was available *ad libitum* for seven days until the start of scheduled feeding. Water was unrestricted throughout the entire experiment. Wheel-running data were collected in 5-min bins using the Vital View data collection system (MiniMitter, Bend, OR) following standard protocols (Amy *et al.,* 2000; Yan *et al.,* 2003; Castillo *et al.*, 2004). Activity data were analyzed by Actiview (MiniMitter).

After the initial period of *ad libitum* feeding on a 12:12 light-dark cycle, food availability was decreased to 10 hours (food available from 4 hours after light on until 2 hours after light off). Subsequently, food available was reduced to 6 hours by removing food 1 hour earlier every 3 days (Castillo *et al.*, 2004). After 11–18 days on the 6-hour feeding schedule under a 12:12 LD cycle, the mice were euthanized for blood collection. One ENTR mouse died during the scheduled feeding experiment.

Once scheduled feeding was initiated, bedding was changed daily at the time of food removal to prevent animals from hoarding food. Cages were changed weekly. Mean daily food usage was measured after food removal starting from the last three days of *ad libitum* feeding to the end of the experiment. Food usage (a daily change in the weight of food in the feeder), instead of food eaten, was used because the actual amount of food eaten was difficult to determine accurately in our cage setup. Mean amount of food used (± SE) under scheduled feeding was not different from that used under *ad libitum* feeding (ENTR line, 5.42±0.08 and 5.64±1.44 g, respectively, t_{70} =1.29, p>0.1; NON-ENTR line, 6.38±0.12 and 6.70±0.21 g, respectively, t_{70}=1.29, p>0.1).

Animal care and experimental procedures were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (protocol #05–44).

Procedure for obtaining blood samples

For animals under *ad libitum* feeding, 11 to 13 randomly chosen mice of each line were euthanized at different zeitgeber (ZT) times (ZT0 is defined as the start of lights on). Animals in the room with 6 am–6 pm light on were sacrificed at ZT1, ZT6 and ZT11, while animals in the room with a reversed light-dark cycle were sacrificed at ZT13, ZT18 and ZT23. For animals under scheduled feeding, 6 randomly chosen mice of each line were sacrificed at different times of the 24-hour day (ZT1, ZT6, ZT11, ZT13, ZT18, and ZT23). The mice were given an overdose injection (intraperitoneal-IP) of a sodium pentobarbital solution (10–15 mg/kg). When slowed breathing and no toe-pinch reflex were observed, their chests were opened and blood samples were collected from the left ventricle using a 22G needle attached to a 3cc syring. When mice were in darkness, the sampling procedures were carried out under a red light. Whole blood from each mouse was put into two vials. One vial contained K_3-EDTA (1.735 mg/ml) and after centrifugation the plasma was stored at −20 °C for the leptin blood assay. Another vial contained K_3-EDTA (1.25 mg/ml) and aprotinin (500U). After centrifugation, the plasma was acidified by adding 100 µl of 1 mol/L HCL per ml of collected plasma and stored at −80 °C for the active ghrelin blood assay. All the mice were weighed just before they were sampled.

Biochemical analysis

Mouse plasma leptin concentrations were determined with Mouse Leptin Enzyme Linked Immunosorbent Assay (ELISA) Kits (Linco Research, St Charles, MO, USA). The specificity of mouse leptin is 100%. After the microtiter assay plate was washed with the washing buffer, the plasma samples were placed in testing wells (10 μl plasma in each well), which were coated with the pre-tittered capture antibodies that immobilized leptin molecules. Then the mouse anti-leptin primary antibody was added to each well. The plate was incubated for 2 hours at room temperature. Subsequently, the wells were washed and the pre-tittered biotinylated anti-mouse antibody, which acted as the secondary antibody, was added. The plate was then incubated again for 1 hour at room temperature. The wells were washed again and the pre-tittered streptavidin-horseradish peroxidase (HRP) was added. After 30 min of incubation, the substrate 3,3,5,5-tetramethylbenzidine was added under shaded conditions to monitor the enzyme activities by converting the enzyme into chromogenic signal. After acidification with stop solution, the absorbance units were measured spectrophotometrically by the increased absorbance at 450 nm, corrected for the absorbance at 590 nm. Each sample was measured in duplicate. Coefficients of variances (CV) ranged from 1.2% to 1.7% within runs and from 3.1% to 4.6% between runs.

Mouse plasma active ghrelin levels were determined with Active Ghrelin ELISA Kits (Linco Research, St Charles, MO, USA). The specificity for rat/mouse active ghrelin is 100%. The plasma samples were placed in testing wells (50 μl plasma in each well) coated with mouse monoclonal antibody directed to the N-terminal of active

ghrelin. Then, the plate was incubated for 2 hours at room temperature. Subsequently, the samples were washed, and diluted HRP conjugated mouse monoclonal antibody directed to the C-terminal of active ghrelin was added to the wells. The plate was then incubated again for 1 hour at room temperature. Then, the samples were washed and 3,3,5,5-tetramethylbenzidine as substrate for HRP was added to the wells. The plate was incubated for an additional 30 minutes at room temperature in the dark. After the reaction, the stop solution was added, and the absorbance unit was read spectrophotometrically at 450 nm. Each sample was measured in duplicate. CVs ranged from 3.5% to 5.5% within runs and from 2.8% to 4.2% between runs.

In order to validate that plasma leptin and active ghrelin levels measured on separate ELISA microplates could be directly compared, we performed additional hormone blood assays with a subset of samples taken from *ad libitum* fed mice and scheduled fed mice and quantified them on the same microplates. Regression analysis between the results (Fig. 1) revealed that the different assays provided comparable results.

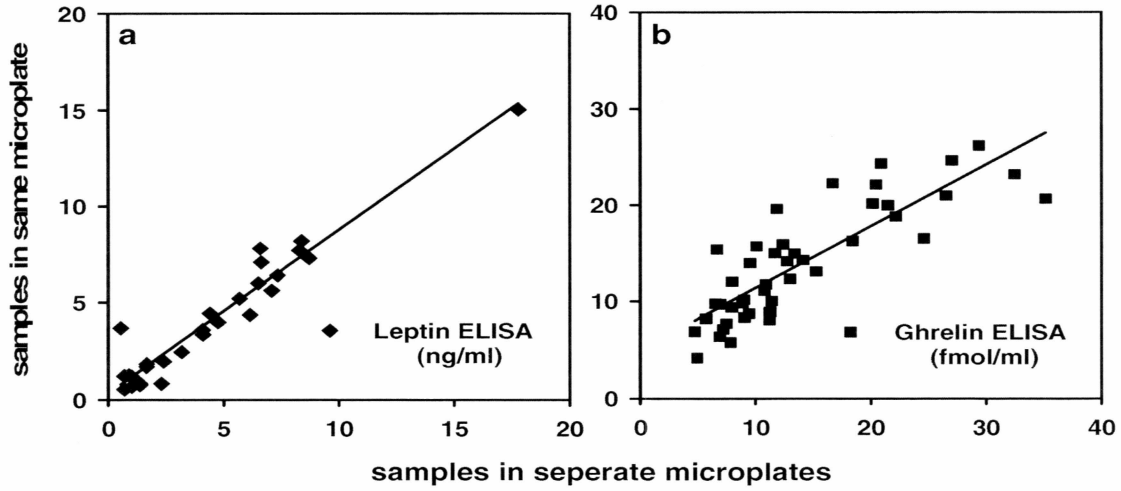

Figure 1 Validation of interassay variances of plasma leptin (a) and active ghrelin (b). X-axis shows ELISA results of samples under different feeding conditions measured in separate microplates. Y-axis shows ELISA results of the same samples under different feeding conditions analyzed in the same microplates (for leptin, n=29, r=0.96; for active ghrelin, n=45, r=0.84).

Statistics

Plasma hormone concentrations and body weight are presented as means±S.E.M.; $p<0.05$ was considered statistically significant. Body weight of ENTR and NON-ENTR mice and hormone concentrations at daytime and nighttime were compared using t-tests for independent samples. Because body weights of the two lines of mice are significantly different (NON-ENTR mice are significantly heavier than ENTR mice) differences of the hormone levels between the two mice lines under different experiment conditions were tested by using analysis of covariance (ANCOVA) with mouse line, treatment, time point, and their interactions as factors, and body weight as a covariate. In order to meet normality and homogeneity of variance assumptions, $\log_{10}$ transformed data for hormone concentrations and body weight were used. Parallelism of slopes between factors and

covariate were tested. When covariate and factors interactions were not significant, only the factors, interactions between factors and the covariate were kept in the ANCOVA model. However, if one of factors and covariate interaction was significant, all the factors, interactions between factors, interactions between factors and covariate, and the covariate were kept in the model. Linear regressions were performed between leptin levels and body weight under different feeding conditions for the two lines of mice because the body weight and treatment interaction effect was significant for leptin levels. Differences between hormone levels at any two time points were tested by the Tukey studentized range test method. SAS software was used for data analysis.

Results

Body weight

Under *ad libitum* feeding, mouse body weight was significantly different between ENTR and NON-ENTR lines. NON-ENTR mice on average were 10.4 g heavier than ENTR mice at the age of 60–70 days, right before they were sampled (i.e., 38.6±0.5 g vs. 28.2±0.4 g; NON-ENTR line vs. ENTR line, $t_{147} = 17.88$, $p<0.0001$).

Under scheduled feeding, body weight of ENTR mice was significantly different from that of NON-ENTR mice after 11 to 18 days of 6-hour food availability. NON-ENTR mice on average were also 10.4 g heavier than the ENTR mice at the age of 60–70 days, right before they were sampled (i.e., 37.3±0.7 g vs. 26.9±0.5 g; NON-ENTR line vs. ENTR line, $t_{70} = 12.1$, $p<0.0001$).

For both ENTR and NON-ENTR mice, no significant difference between their body weight under *ad libitum* feeding and scheduled feeding was observed (ENTR line, t_{107} =1.88, p>0.05; NON-ENTR line, t_{110} =1.54, p>0.1).

Plasma leptin concentrations

No clear circadian patterns in plasma leptin concentrations were found in ENTR and NON-ENTR mice under ad libitum feeding in a 12:12 LD cycle (Fig. 2a), while robust circadian rhythms were observed under scheduled feeding (Fig. 2b), which explains the significant treatment by time point interaction effect ($F_{5,174}$=13.16, p< 0.0001). The ENTR and NON-ENTR mice were only significantly different for plasma leptin levels at ZT13 under ad libitum feeding (Fig. 2a), which is reflected in the non-significant mouse line effect ($F_{1,174}$=1.37, p>0.1). Body weight had a large and significant effect on plasma leptin levels (body weight effect; $F_{1,174}$=24.80, p<0.0001). Plasma leptin concentrations correlated positively and significantly with body weight both in ENTR mice (*ad libitum* feeding, r=0.65, n=72, p<0.0001; scheduled feeding, r=0.55, n=30, p<0.005) and NON-ENTR mice (*ad libitum* feeding, r=0.59, n=69, p<0.0001; scheduled feeding, r=0.55, n=35, p<0.001). The slopes of the regression were similar for ENTR (3.86) and NON-ENTR (3.24) mice under *ad libitum* feeding and NON-ENTR mice under scheduled feeding (3.53), but lower than the ENTR mice (6.42) under scheduled feeding (Fig. 3). The relationship between plasma leptin concentration and mice body weight changed in ENTR mice under scheduled feeding because mice

with lower body weight had relatively lower leptin levels. The significant treatment by body weight interaction effect ($F_{1,174}$=6.11, p<0.05) probably reflects this difference.

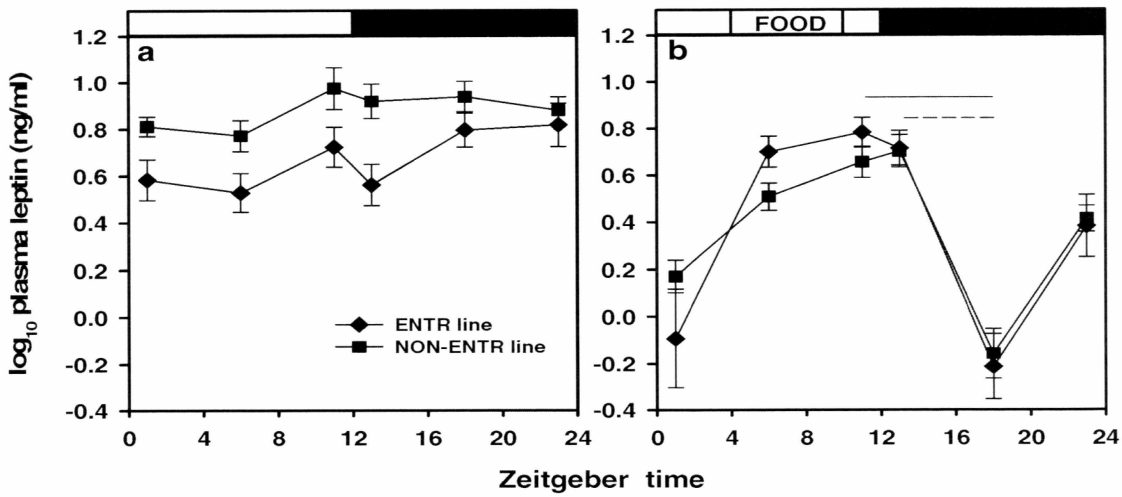

Figure 2 Diurnal rhythms of plasma leptin concentrations (means ± SEM) (log_{10} transformed) of ENTR and NON-ENTR mice under *ad libitum* feeding (a) and scheduled feeding (b) in a 12:12 LD cycle. For *ad libitum* feeding, mice were sampled at ZT1 (n=12 vs. n=9; ENTR mice vs. NON-ENTR mice), ZT6 (n=12 vs. n= 14), ZT11 (n=12 vs. n=11), ZT13 (n=11 vs. n=12), ZT18 (n=13 vs. n=12), ZT23 (n=12 vs. n=11). For scheduled feeding, mice were sampled at ZT1 (n=6 vs. n=6; ENTR mice vs. NON-ENTR mice), ZT6 (n=4 vs. n=6), ZT11 (n=4 vs. n=6), ZT13 (n=6 vs. n=6), ZT18 (n=6 vs. n=5), ZT23 (n=4 vs. n=6). * Significant difference (Tukey, p<0.05) between ENTR and NON-ENTR mice at the same time point. —— Significant difference (Tukey, p<0.05) between peak (ZT11) and trough (ZT18) for ENTR mice. -------Significant difference (Tukey, p<0.05) between peak (ZT13) and trough (ZT18) for NON-ENTR mice. ZT0 is defined as the time of lights on.

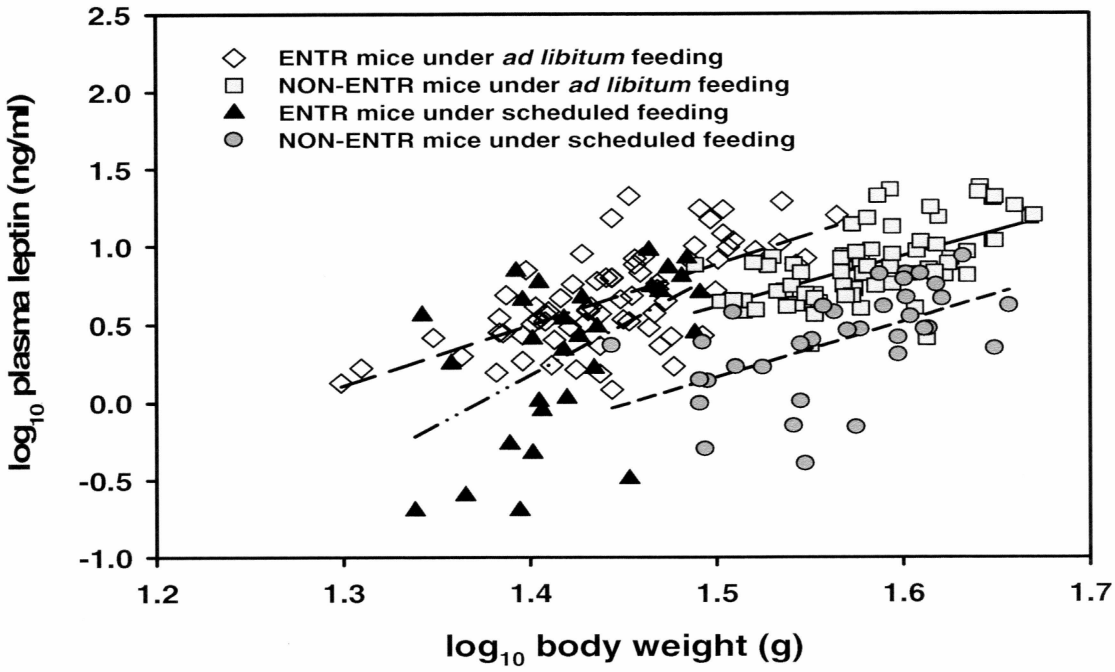

Figure 3 Linear regression between plasma leptin concentrations (log_{10} transformed) and mouse body weight (log_{10} transformed) of ENTR and NON-ENTR mice under *ad libitum* feeding and scheduled feeding.

No effects of time point ($F_{5,174}$=0.52, p>0.5), treatment by mouse line interaction ($F_{1,174}$=0.43, p>0.5), mouse line by time point interaction ($F_{5,174}$=0.65, p>0.5), treatment by time point interaction ($F_{5,174}$=1.91, p>0.05), mouse line by body weight interaction ($F_{1,174}$=1.89, p>0.1), and time point by body weight interaction ($F_{5,174}$=0.48, p>0.5) were found.

After scheduled feeding, average plasma leptin concentrations calculated over the 24-hour day decreased significantly in both lines of mice (Fig. 4), which accounts for the significant treatment effect ($F_{1,174}$=5.02, p<0.05). For ENTR mice, leptin decreased

compared to *ad libitum* feeding by 42.0% (t_{100}=3.42, p<0.001), and for NON-ENTR mice, leptin decreased by 65.7% (t_{102}=7.71, p<0.0001).

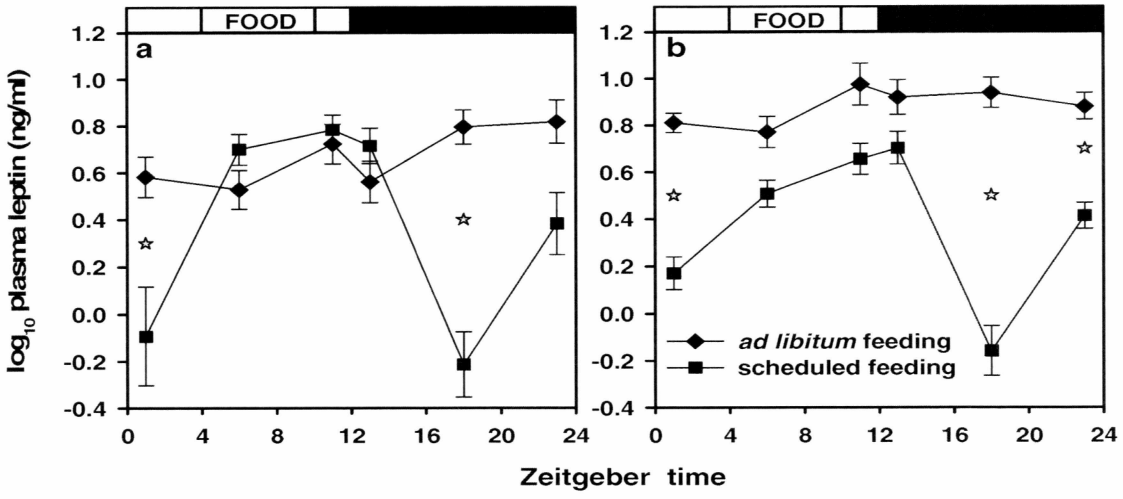

Figure 4 ENTR (a) and NON-ENTR (b) mouse plasma leptin concentration (log_{10} transformed) rhythms comparison between *ad libitum* feeding and scheduled feeding. * Significant difference (Tukey, p<0.05) between *ad libitum* feeding and scheduled feeding at the same time point. ZT0 is defined as the time of lights on.

Plasma active ghrelin concentrations

ENTR mice showed significant circadian rhythms in plasma active ghrelin concentrations with peak levels at ZT13 and ZT18 and trough levels at ZT6 and ZT11 under *ad libitum* and scheduled feeding (time point effect; $F_{1,158}$=6.11, p<0.0001), respectively (Fig. 5). The change in timing of the peaks and troughs accounts for the significant treatment by time point interaction effect ($F_{5,158}$=3.36, p<0.01). NON-ENTR mice did not reveal significant circadian variation in plasma active ghrelin levels under either feeding condition (Fig. 5). These results account for the significant mouse line by time point interaction ($F_{5,158}$=6.49, p<0.0001) and mice line by treatment by time point

interaction ($F_{5,158}$=2.41, p<0.05) effects. The overall plasma active ghrelin levels for both feeding conditions were not different between the ENTR and NON-ENTR mice (mouse line effect; $F_{1,158}$=0.11, p>0.5).

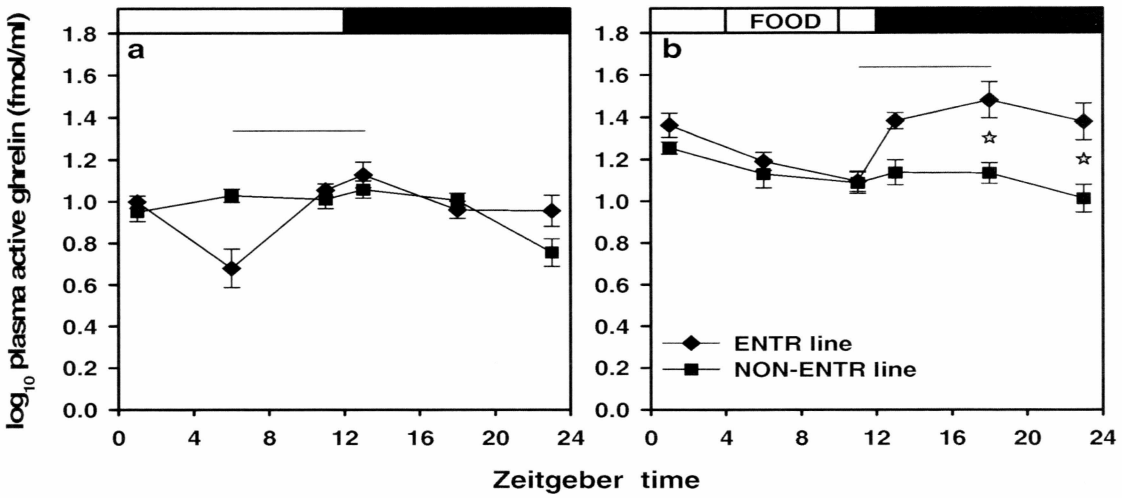

Figure 5 Diurnal rhythms of plasma active ghrelin concentrations (means ± SEM) (log_{10} transformed) of ENTR and NON-ENTR mice under *ad libitum* feeding (a) and scheduled feeding (b). For *ad libitum* feeding, mice were sampled at ZT1 (n=14 vs. n=12; ENTR mice vs. NON-ENTR mice), ZT6 (n=6 vs. n=8), ZT11 (n=12 vs. n=12), ZT13 (n=12 vs. n=13), ZT18 (n=8 vs. n=6), ZT23 (n=6 vs. n=5). For scheduled feeding, mice were sampled at ZT1 (n=6 vs. n=6; ENTR mice vs. NON-ENTR mice), ZT6 (n=6 vs. n=6), ZT11 (n=6 vs. n=6), ZT13 (n=5 vs. n=6), ZT18 (n=6 vs. n=5), ZT23 (n=5 vs. n=6). *Significant difference (Tukey, p<0.05) between ENTR and NON-ENTR mice at the same time point. —— Significant difference (Tukey, p<0.05) between ZT13 (peak) and ZT6 (trough) for ENTR mice. ZT0 is defined as the time of lights on.

The covariate body weight affected plasma active ghrelin levels (body weight effect; $F_{1,158}$=8.15, p<0.01), but none of the covariate and factor interaction effects were significant (data not shown). Plasma active ghrelin concentration correlated negatively with mouse body weight.

After scheduled feeding, average plasma active ghrelin concentration calculated over the 24-hour day increased significantly in both lines of mice (Fig. 6; treatment effect; $F_{1,158}$=116.88, p<0.0001). For ENTR mice, ghrelin increased compared to *ad libitum* feeding by 121.0% (t_{90}=7.58, p<0.0001) and for NON-ENTR mice, ghrelin increased by 42.6% (t_{89}=4.29, p<0.0001). The difference between the lines in the amount of decrease resulted in a significant treatment by mouse line interaction effect ($F_{1,158}$=17.21, p<0.0001).

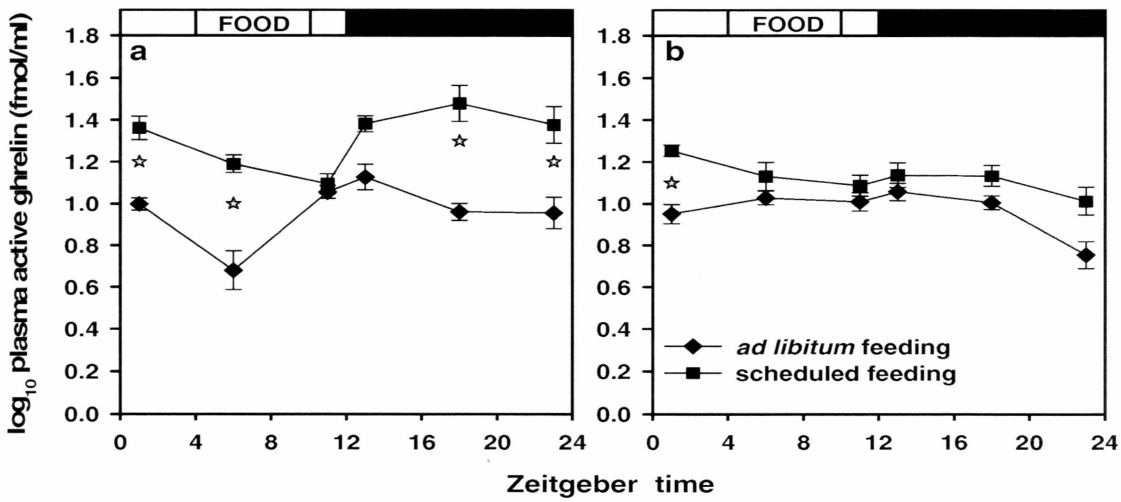

Figure 6 ENTR (a) and NON-ENTR (b) mice plasma active ghrelin concentration (log_{10} transformed) rhythms comparison between *ad libitum* feeding and scheduled feeding. * Significant difference (Tukey, p<0.05) between *ad libitum* feeding and scheduled feeding at the same time point. ZT0 is defined as the time of lights on.

Behavioral entrainment to scheduled feeding

The hourly activity profiles for wheel-running activity of ENTR and NON-ENTR mice were similar under scheduled feeding in a light-dark cycle (Fig. 7). Both of them showed distinct FAA and light entrainable activity (LEA) during the dark period. The

activity profiles of FAA and LEA showed similar patterns although the overall activity level (number of wheel revolution per day) of ENTR mice was significant lower than that of NON-ENTR mice (t_{64}=4.35 p<0.0001; Fig. 7). Both lines of mice increased their wheel running activity sharply about 2 hours before the start of food availability and decreased their activity in the hour following the start of food availability (Fig. 7). For FAA, both lines of mice reached maximum activity during the hour before the start of food availability and no difference of the activity level between them at this time was found. For LEA, both lines increased wheel-running activity immediately after light off and decreased their activity sharply 4 hours later (Fig. 7). Then for ENTR mice, wheel-running activity reached the lowest level through the first hour after lights on. For NON-ENTR mice, activity levels continued to decrease until the lowest level was reached around the end of light off. The two lines of mice reached the maximum activity of LEA at the same time point (between ZT13 and ZT14), although the activity level at that time in ENTR mice was lower than that of NON-ENTR mice.

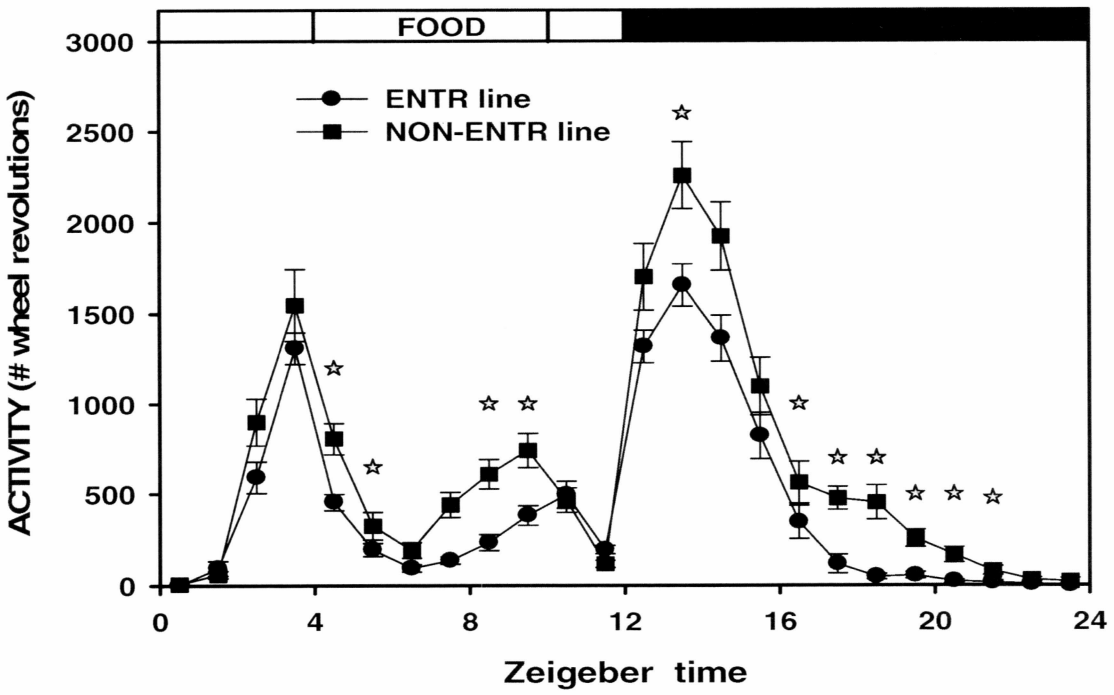

Figure 7 Hourly activity profiles of wheel running of ENTR and NON-ENTR mice under 6 hours of food availability in a 12:12 light-dark cycle (hourly mean ± SEM) (n=5–6). The black bar indicates the dark period. The box with "Food" indicates the time period of food available (ZT4 to ZT10). *Significant difference (Tukey, $p<0.05$) between ENTR and NON-ENTR mice at the same time point. ZT0 is defined as the time of lights on. This figure uses wheel running activity data of the 4th through the 9th day under 6 hours of food availability in a 12:12 LD cycle.

Discussion

I hypothesized that the leptin and active ghrelin hormones provide direct or indirect signals for entrainment of the SCN to SF, because of their important role in energy homeostasis. In this context, I will discuss the results on circadian patterns in plasma levels of these hormones of our ENTR and NON-ENTR mice under *ad libitum*

feeding and SF first for leptin, then for active ghrelin, and lastly for the comparison of the circadian patterns of these hormones.

Circadian plasma leptin levels under different feeding conditions

Previous reports have shown that plasma leptin levels correlate positively and significantly to body weight (Caro *et al.,* 1996; Considine *et al.,* 1996; Pelleymounter *et al.,* 1995; Frederich *et al.,* 1995; Korhonen and Saareha, 2005). Our mice showed the same patterns, although the relationship between leptin levels and body weight in ENTR mice changed after scheduled feeding (Fig. 3). The ENTR mice are significantly smaller than NON-ENTR mice. In order to survive, ENTR mice require a gradual shortening of the food availability period when scheduled feeding is initiated (unpublished observations), probably because they have lower fat reserves compared to the NON-ENTR mice. This shows that scheduled feeding may be more taxing on the ENTR mice and may explain the relatively lower leptin levels in the smallest ENTR mice under scheduled feeding.

In mice, rats, and humans, plasma leptin levels are generally higher at night compared to the daytime (Boden *et al.,* 1996; Laughlin and Yen., 1997; Schoeller *et al.,* 1997; Saad *et al.,* 1998; Ahren, 2000; Tolle *et al.,* 2002). We found similar patterns in our mice, although they were not significant. For humans, the rise in leptin circulation levels during the night is thought to reflect the accumulated energy throughout the day (Laughlin and Yen, 1997; Saad *et al.,* 1998). For nocturnal rodents, the higher leptin

levels during the dark phase seem responsible for the gradual decline of feeding throughout the night (Ahren, 2000; Tolle *et al.*, 2002; Kalra and Kalra, 2003).

Scheduled feeding had a strong impact on plasma leptin rhythms. Under scheduled feeding, ENTR and NON-ENTR mice showed peak leptin levels at the end of the day or at the start of the night, respectively, and trough leptin levels in the middle of the night, which tended to be opposite to the rhythm observed under *ad libitum* feeding (Figs. 2, 4). This result was consistent with findings in rats exposed to 12-hour food availability during the day. These rats show opposite plasma leptin patterns compared to those during *ad libitum* feeding (Bodosi *et al.,* 2004). When food availability is restricted to 2 hours daily in rats, diurnal rhythms of leptin are entrained and their peak levels shift to around mealtime (Martinez-Merlos *et al.,* 2004). Mice restricted to 4 hours of daytime feeding generate an extra leptin surge at daytime (Ahima *et al.,* 1998). Collectively, these studies reveal that plasma leptin can be entrained by mealtime in a light-dark cycle independent of the master circadian clock in the SCN (Schoeller *et al.,* 1997; Sanchez *et al.,* 2004), which is still entrained to the light dark cycle (Castillo *et al.*, 2004). However, some studies show that under *ad libitum* feeding, the SCN controls plasma leptin rhythms because SCN lesions abolish this circadian rhythm in rats (Kalsbeek *et al.,* 2001).

Some studies suggest that the leptin rhythm is controlled by circulating glucocorticoid rhythms (Dagogo-Jack *et al.,* 1997; Newcomer *et al.,* 1998; Elimam and Marcus, 2002). However, adrenalectomized rats with constant corticosterone release through implanted pellets have the identical leptin rhythm compared to the control group with the normal diurnal peak of corticosterone (Kalsbeek *et al.,* 2001), which reveals that

circadian variation in plasma corticosterone cannot explain the circadian pattern of rat plasma leptin levels. In both rats and humans, a meal-induced rise in insulin level contributes to the nocturnal rise in leptin (Saladin *et al.,* 1995; Boden *et al.,* 1996; Laughlin *et al.,* 1997; Schoeller *et al.,* 1997; Saad *et al.,* 1998). Studies in mice also suggest that under *ad libitum* feeding, the nocturnal increase in insulin and leptin levels are highly correlated with the rise in insulin preceding the rise in leptin (Ahren, 2000). During fasting, the reduction in leptin also correlates to a reduction in insulin (Ahren, 2000). These findings suggest that insulin is the most important regulator of leptin.

In our scheduled feeding study, wheel running FAA and LEA were clearly demonstrated (Fig. 7), which was consistent with previous findings (Castillo *et al.*, 2004). Although the total daily activity level of the ENTR mice was lower than that of the NON-ENTR mice, this difference was mainly due to differences in activity levels during LEA. The average activity patterns of the ENTR and NON-ENTR mice during FAA were very similar and not significantly different. To what extent the smaller body weight of the ENTR mice contributed to a decreased activity level under scheduled feeding, although these mice under *ad libitum* feeding are more active (Bult *et al.*, 1993) or have similar activity levels (Castillo *et al.*, 2005), remains to be elucidated. Under scheduled feeding, plasma leptin levels increased from ZT1 to ZT6 (2 hours after the start of food availability), but our time resolution was insufficient to determine whether this increase coincided with the increase in FAA activity. However, it is unlikely that this rise in leptin was responsible for the initiation of FAA because daily leptin administration does not produce FAA in rats (Martinez-Merlos *et al.,* 2004).

Circadian plasma active ghrelin levels under different feeding conditions

This is the first report of plasma active ghrelin circadian rhythms in mice. Most studies on ghrelin so far refer to total plasma ghrelin, which includes active and inactive forms, but only active ghrelin has biological activity (Horvath *et al.,* 2001; Yamaguchi *et al.,* 2003; Dornonville *et al.*, 2005). The relationship between active and inactive ghrelin is poorly understood. Although some studies report that active ghrelin is proportional to total ghrelin and that the ratio of active ghrelin versus total ghrelin is similar in different individuals (Date *et al.,* 2002; Dornonville *et al.*, 2005), others do not find a fixed ratio of active to total ghrelin (Casanueva and Dieguez, 2004).

An inverse correlation between ghrelin levels and body weight has been reported for mice (Korhonen and Saareha, 2005) and humans (Tschop *et al.*, 2001). In our mice, body weight affected plasma active ghrelin levels negatively. Studies demonstrate that body weight loss substantially elevates ghrelin levels in humans and other animals (Wisse *et al.,* 2001; Cummings *et al.,* 2002; Foster-Schubert *et al.,* 2005). Ghrelin may mainly be stimulated by negative caloric balance.

ENTR mice had robust plasma active ghrelin circadian patterns under *ad libitum* and scheduled feeding, while for the NON-ENTR mice no significant patterns were found (Figs. 5, 6). Studies in humans and sheep suggest that ghrelin secretion can be observed before each meal and its level decreases quickly 1h after the meal (Cummings *et al.,* 2001; Sugino *et al.,* 2002). Because we focused on circadian ghrelin rhythms, we did not monitor the detailed feeding behavior of our mice. Detailed feeding pattern

differences between our mice and the effects of these patterns on the plasma active ghrelin circadian rhythms remain to be investigated in our mice.

Plasma ghrelin levels generally peak at midday (Kalra *et al.*, 2003; Bodosi *et al.*, 2004; Korhonen and Saareha, 2005), although plasma active ghrelin in the ENTR mice peaked at the start of the dark period (Fig. 5). In rats, a second ghrelin rise before the time of light onset has been reported (Murakami *et al*; 2002). Whether the differences between our mice and these studies can be explained by our quantification of active ghrelin instead of total ghrelin or our choice of time points remains to be elucidated.

Under scheduled feeding, the ENTR mice had high levels of plasma active ghrelin during the 12 hours preceding the food availability period (Figs. 5, 6). During the food availability period (ZT4 to ZT10), active ghrelin decreased gradually and reached trough levels at ZT11. These data were qualitatively similar to rats under 4-hrs of scheduled feeding, which revealed that ghrelin rises two hours preceding the start of scheduled feeding time, peaks half an hour prior to food availability, and decreases gradually to the baseline levels during the food availability period (Drazen *et al.*, 2006). A reasonable interpretation of the increasing ghrelin level before a scheduled meal is that animals need to make metabolic adjustments in order to eat their full day's calories over a short period of time (Woods, 1991; Woods and Strubbe, 1994; Drazen *et al.*, 2006).

Where ghrelin acts to stimulate eating is uncertain. In rats, after 48 hrs of food deprivation, subdiaphragmatic vagotomy prevents the significant rise in ghrelin levels observed in the sham control group (Williams *et al.*, 2003). This study demonstrates that the vagus nerve plays an important role in the secretion of ghrelin. Other studies also

suggest that ghrelin stimulates eating via vagus nerve-mediated abdominal action (Asakawa *et al.,* 2001; Date *et al.,* 2002, 2005; le Roux *et al.,* 2005). However, a recent study testing the effects of subdiaphragmatic vagal deafferentation on ghrelin-induced eating suggests that abdominal vagal afferents are not involved in ghrelin-induced eating (Arnold *et al.,* 2006). Instead, the possible eating stimulating effect of ghrelin might arise from the brain rather than from the periphery (Arnold *et al.,* 2006).

Relationship between circadian plasma leptin and active ghrelin levels after scheduled feeding

Interestingly, for both ENTR and NON-ENTR mice, active ghrelin showed reversed circadian rhythms with leptin, which was most pronounced under scheduled feeding (Figs. 2, 5), while another study shows the reversed leptin and ghrelin circadian rhythms under *ad libitum* feeding and scheduled feeding (Bodosi *et al.,* 2004). A study on rats suggests that the plasma ghrelin rhythm is more-or-less in phase with leptin under *ad libitum* feeding except for the rise just before the onset of dark phase (Sanchez *et al.,* 2004).

Compared to *ad libitum* feeding, scheduled feeding decreased the overall concentration of plasma leptin and at the same time increased the overall concentration of plasma active ghrelin significantly in both ENTR and NON-ENTR mice. Rats with 4-hours of scheduled feeding during the light period also show lower leptin concentrations and higher active ghrelin concentrations (Drazen *et al.*, 2006). Our results were very much in line with the studies on animals under transient fasting (Bagnasco *et al.,* 2002;

Kohonen and Saareha, 2005). However, transient fasting usually significantly decreases the animal's body weight (Kohonen and Saareha, 2005). Rats with 4-hours daily food restriction also have lowered body weight (Drazen *et al.*, 2006). In our study, the body weight of the ENTR and NON-ENTR mice under *ad libitum* and scheduled feeding was not significantly different.

Because scheduled feeding did not decrease body weight of the ENTR and NON-ENTR mice, the decrease in leptin secretion is not related to the content of fat tissue during this process, which is consistent with transient fasting studies (Schwartz *et al.*, 1997). The main regulator of leptin secretion during fasting and scheduled feeding could be the activity level of the sympathetic nervous system (SNS) in white adipose tissue (WAT) because increased activity of the SNS in WAT decreases leptin gene expression and leptin production (Rayner and Trayhurn, 2001). Sympathetic stimulation of WAT is increased during fasting (Kohonen and Saareha, 2005). This response may also occur during scheduled feeding.

Leptin has been suggested to be the major inhibitor of ghrelin through NPYergic signaling in the hypothalamus (Kalra *et al.,* 2003; Ueno *et al.,* 2004; Kalra *et al.,* 2005). Under normal energy balanced conditions, relatively high levels of leptin could constrain secretion of ghrelin. During fasting or scheduled feeding, the lowed leptin tone may promote secretion of ghrelin (Kalra *et al.,* 2003). Leptin and ghrelin are antagonistic hormones, having anorexigenic and orexigenic effects, respectively. Their plasma levels respond to scheduled feeding in an inverse manner, they may regulate hypothalamic peptidergic systems together by inducing more NPY and AgRP expression in the PVN,

which contributes to energy saving and prevents further energy deficits (Shintani *et al.*, 2001).

Conclusions

In conclusion, the plasma leptin and active ghrelin circadian rhythms reported here for mice generally agree with previous findings for other mammals. Some of the differences between this study and other published reports on ghrelin may be due to our measuring active ghrelin, while others quantified total ghrelin. Under *ad libitum* feeding and scheduled feeding, the plasma active ghrelin circadian rhythms of ENTR mice were very robust, while no significant circadian patterns were found in the NON-ENTR mice. This is in agreement with the NON-ENTR mice (big nest-builders) having less robust circadian organization of wheel running activity (Bult *et al.,* 2001) and body temperature (Castillo *et al.*, 2005), including more fragmentation of the circadian rhythm and more activity during the light period, than the ENTR mice (small nest-builders) (Bult *et al.,* 1993, 2001). The high amplitude plasma active ghrelin circadian rhythms may provide a signal for the ENTR mice to entrain to scheduled feeding as reported in Castillo *et al.* (2004), and the non-significant active ghrelin circadian rhythms observed in the NON-ENTR mice may not provide sufficiently powerful timing cues for entrainment to scheduled feeding of most of these mice. This interpretation is in agreement with a recent study that reveals that ghrelin phase shifts the SCN in vitro (Yannielli *et al.*, 2007). Plasma leptin levels were not different between the ENTR and NON-ENTR mice and leptin is not expected to influence entrainment to scheduled feeding.

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